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"CHARACTERIZATION OF BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI FROM EDIBLE GREENS"

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Chapter 1
General Introduction

The use and consumption of leafy greens can be traced back to the beginning of recorded history. Microorganisms are abundant in the natural world. They can be found in the most incompatible environments across the biosphere's ecosystems. They were on this planet for at least 3.7 billion years, 3.0 billion years before plants and animals appeared. They are the ancestors of all living species and account for roughly 60% of all biomasses on the planet. Microorganisms have contributed to the growth of industry for over a century by manufacturing a variety of important goods (Demain and Adrio, 2008).

Secondary metabolites are small molecules produced by microorganisms in response to evolutionary survival pressures and challenges posed by specific ecological niches. They are not required for their growth but are produced in response to evolutionary survival pressures and challenges posed by specific ecological niches (Cole and Schweikert, 2003).

Microorganisms are major sources of bioactive natural compounds with enormous promise for drug development, industrial applications, and agricultural applications (Keller *et al.,* 2005). Most of the antibiotics and therapeutic medications created today are originated from filamentous fungus, actinomycetes, and bacteria, secondary metabolites from these taxonomic groups have had a lot of success. Natural products are thought to make up around a third of all commercially marketed medications (Elsewijk and Irth, 2003).

Endophytic microorganisms are those that are prevalent in some parts or throughout a plant and spend their whole life cycle or a portion of it without creating disease signs (Schulz and Boyle 2005). Debary first coined the term "endophyte" in 1866. Endophytes have adapted to the plant microenvironment through genetic diversity, which includes DNA acquisition from some plants (Germaine *et al.* 2004). Endophytes began manufacturing plant metabolites or their precursors as a result of this adaptation and genetic material acquisition (Zhang *et al.* 2006). Because just a few plant species have been examined for endophytes, there is a lot of room for endophytic microorganisms and their bioactive compounds to be discovered, particularly in traditional herbs. Endophytic microorganisms are vertically transmitted, co-evolve with their hosts, and have parasitic to strongly mutualistic relationships (Porras-Alfaro and Bayman, 2011). They contribute to plant health by giving resistance to the hosts against various biotic and abiotic stressors (Kharwar *et al.,* 2008; Gond *et al.,* 2010).

Alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavonoids, phenols and phenolic acids, and peptides are some of the functional metabolites produced by endophytes. These compounds can also be used as structurally unique metabolites with a wide range of biological properties.

Endophytes of fungi, particularly ascomycetes, are abundant, but basidiomycetes, deuteromycetes, and oomycetes are uncommon. Endophytic fungi and their ecological roles are extremely diverse, as it is the chemical diversity of their secondary metabolites, which hold potential for medicinal or agrochemical applications when examined in various bioassays (Strobel *et al.,* 2004). Since the discovery of the most important bioactive compound, paclitaxel (taxol), from the endophytic fungus Taxomyces andreanae in 1993, many scientists have been interested in studying fungal endophytes as potential sources of novel natural agents for exploitation in the pharmaceutical industry, agriculture, and environmental applications (Zhao *et al.,* 2010).

Human health is becoming increasingly complicated as a result of present lifestyles, changes in community ethics, and the loss of traditional wisdom in rural areas (Maikhuri *et al.,* 2004). Currently, approximately 80% of adults and 56% of children do not consume the recommended number of vegetables (FAO/WHO, 2001), which promotes diet-related problems such as diabetes, obesity, high blood pressure, heart disease, and mental decline (Iyer *et al.,* 2012). Consumption of leafy greens rich in bioactive components aids in the maintenance of a balanced diet and can be cultivated with knowledge; yet, the usage of green leaves has been ignored and underutilized (Shei, 2008). Limited information is available on the medicinal properties associated with leafy vegetable consumption in South India. Most ethnobotanical studies on leafy vegetables concentrate on wild and weedy species (Palozza and Krinsky 1992). As a result, any knowledge on plants used by native communities must be recorded and preserved as soon as possible.

In this work, endophytic fungi were isolated and identified from a variety of used and underutilised green leafy vegetables from a specific location in Kodagu District.

OBJECTIVES OF THE STUDY

1. Isolation and identification endophytic fungi in edible leafy greens.
2. Biological activity of Endophytic Fungi.
3. Molecular identification of selected endophytic fungi.
4. Characterization of bioactive compounds of endophytic fungi.

CHAPTER 2

Review of literature

Food is an essential component for the life of humans as well as the maintenance of their health. The absence of disease is the most important aspect of health, which can be achieved when all a person's bodily systems are working harmoniously. A healthy diet is the best tactic for positive effects on health achieved by intake of limited calories, available easily in nature in the form of plants. Leaves are the primary site of photosynthesis in plants and are generally the most nutrient dense and perishable of vegetables. The colour, flavour, and therapeutic benefits of leaves, particularly dark green leaves, are well known. Leaves also contain relatively high levels of minerals (for example, iron, magnesium, and calcium), enzymes (protein), and secondary metabolites (for example, carotenes and xanthophylls) (Gallaher and Schneeman, 2001). Since they offer additional health advantages on top of their basic nutritional value, leafy greens are categorised as a type of food known as a functional food. In terms of the human food chain, plant leaves are a main producer, and hence have a high nutritional value. The complex chemical compounds that are produced in plant leaves contain molecules that act as antioxidants, which protect the plant from the damaging effects of oxidative sun radiation. The consumption of leafy greens in our diet provides an innumerable number of bioactive food components.

Some of the natural compounds produced by the plant hosts are also produced by their endophytes, such as taxol, which is produced by numerous endophytes of *Taxus* spp., such as *Taxomyces andreannae*, *Fusarium* sp., and *Taxomyces* sp (Stierle *et al.*, 1993; Chakravarthi *et al.*, 2008). It was suggested that this could be due to endophyte-host plant genetic recombination during evolution (Tan and Zou, 2001; Strobel and Daisy, 2003). However, in other circumstances, endophytes have been found to create bioactive metabolites independently of the host, showing that they have their own metabolic pathway genes for such compounds (Staniek *et al.*, 2009). Similarly, Friedrich Serturner's discovery of morphine from *Papaver somniferum* in 1806 resulted in considerable testing of natural items as a source of medicines. Natural products are chemical entities that are found in nature and are created by natural systems. They have different pharmacological effects. There are three major groups of natural chemicals. The first group includes compounds that are found in all cells of organisms and play an important role in their metabolism and reproduction. These substances are referred to as main metabolites. They contain common amino acids, nucleic acids, and carbohydrates.

Polymeric substances with high molecular weights, such as lignin, proteins, and cellulose, fit into the second category. The third group includes chemicals that are present in lesser amounts. Secondary metabolites are the name for these substances. Endophytes create secondary metabolites that are comparable to those produced by the host as a result of genetic exchange (Divya and Sathasivasubramanian, 2013).

Secondary metabolites produced by endophytic fungus are a major source of strong bioactive metabolites. Endophytic fungal secondary metabolites have antibacterial, anticancer, antioxidant, insect repellent, cytotoxic, and immune-suppressant effects. A single endophyte can create a variety of bioactive chemicals with different functions. As a result, endophytes are gaining in popularity as a means of exploiting new architectures. Current human medical issues, such as bacterial and fungal infections, drug resistance in pathogenic bacteria, and life-threatening viruses, mandate the development of new therapeutic agents for the effective treatment of human diseases (Zhang *et al.,* 2005). Newman *et al.,* (2016) published a survey of the approved drug lists from 1981 to 2014, stating that bacteria and/or endophytes produced most of the medications. According to the review, over 140 antibacterial chemicals have been developed. 71 were natural product derivatives, 29 were chemically synthesized medications created by modifying existing drugs, and 27 were vaccinations. As a result, endophytes produce unique structures such as alkaloids, flavonoids, benzopyranones, chinones, phenolic acids, quinones, terpenoids, steroids, xanthones, and others (Tan and Zau, 2001) that are beneficial to human health.

A wide variety of plants, including ethnobotanically significant plants, wild plants, and medicinal plants (Sushma *et al.,* 2019), contain bioactive chemicals that can be employed as medications. Primitive people have inherited a wealth of traditional knowledge about plants that grow in their environment that may be used for medicine and food (Dinesh Jadhav, 2006). Edible portions of plants include seeds, roots, tubers, leaves, delicate shoots, flower buds, flowers, and fruits (Nnamani *et al.,* 2010). In comparison to other common vegetables, edible leafy greens provide a lot of nourishment (Orech *et al.,* 2007). Leafy vegetables have high water, cellulose, mineral, and vitamin content and are also termed as potherbs, greens, vegetable greens, leafy greens, or salad greens.

The endophytic fungi are a novel and important microbial resource for producing bioactive compounds and has attracted attention of many researchers on their theoretical study as well as their potential applications besides, studies on diversity of endophytes are scanty especially from India. However, only a few plant hosts have been comprehensively studied

relative to endophytic diversity and biology. Considering the vast plant diversity, it is presumed that a high degree of endophytic fungal diversity also exists and the potential impact of vegetable intake on human health has been extensively explored in nutritional epidemiological studies, showing beneficial associations and suggested that leafy greens deserve special attention with these backgrounds.

Chapter 3

Isolation and identification of endophytic fungi in edible greens

INTRODUCTION

Because there are millions of endophytic fungi occupying millions of unique biological niches and thriving in a variety of environments, they are regarded as an excellent source of bioactive natural compounds (Strobel and Daisy, 2003). Fungal endophyte-infected plants are frequently healthier than endophyte-free plants. This effect could be caused by the creation of phytohormones such IAA, cytokinins, and plant growth promoters (Tan and Zou, 2001). Endophytic fungi have also produced a huge number of biologically useful chemicals, including anticancer medicines, antibiotics, and immunosuppressive substances (Strobel and Daisy, 2003). Endophytes in plants stimulate the production of secondary metabolites in plants that have a wide range of biological functions (Petrini, 1996).

The edible leafy green plants that have been used traditionally for therapeutic purposes and have some documented medicinal efficacy. Because the pharmaceuticals extracted from edible leafy green plants and medicinal plants (herbal drugs) are cost-effective, easily available, and have few adverse effects, they are gaining worldwide interest. Plants are known to host a variety of endophytic microorganisms that produce a variety of substances that could be useful in modern medicine, agriculture, and the pharmaceutical industry (Owen and Hundley, 2004). Endophytic fungi have also produced a huge number of biologically useful chemicals, including new anticancer medicines, antibiotics, and immunosuppressive substances (Kaul *et al.,* 2012; Kharwar *et al.,* 2011). Endophytes in plants encourage the development of secondary metabolites in plants that have a wide range of biological functions (Petrini *et al.,* 2006). Endophytic bacteria have been discovered to create secondary metabolites like those produced by plants, making them a possible source of new chemicals. For example, *Pestalotiopsis microspora*, an endophytic fungus that colonises the Himalayan yew tree *Taxus wallichiana*, was responsible for the discovery of paclitaxel (taxol), the world's first billion-dollar anti-

cancer medicine (Strobel *et al.,* 1996; Gangadevi and Muthumary 2007). As a result, we investigated the secondary metabolism of endophytic fungi from forty edible leafy plants in the hopes of finding a fungal strain capable of producing structurally unique and physiologically active secondary metabolites.

MATERIALS AND METHODS

Study area

Kodagu commonly known as Coorg, established in June 1834. It occupies an area of 4108 sq. km with a humid tropical belt 30-35 percent of the Western Ghats, one of the 8 hottest biodiversity hotspots of the world. Situated to the southwest in Karnataka 12.455º N and 75.957°E with dense forest. Climate is tropical wet during June-October with annual rainfall over 500cm. Due to its environmental condition the main source and income to the people is from agriculture. 40 different fresh leafy greens were collected from different parts of Kodagu District.

Identification of edible plants

The edible leaves were selected based on literature survey with internet, books, journals and from local inhabitants. Standard methods were followed to collect healthy leaves of edible plants and its identification was done using flora books with taxonomic keys (Keshava Murthy and Yoganarasimhan 1990).

Plant collection

Different edible leaves were selected from their natural habitat for the present study. Samples were collected from symptomless healthy and mature plants in sterile zip-lock plastic bags and carefully brought to the laboratory and processed immediately to reduce the chance of contamination.

Surface sterilization of plant materials

Healthy leaves were rinsed in running tap water to remove the soil particles and unwanted debris for one min and then air-dried. The leaves were cut into small segments of 3-5mm in length by using sterile lancet blades under aseptic conditions. These segments were then subjected to surface sterilization by adopting the Vinu and Jayashankar method (2017). The leaf segments were immersed in 70% ethanol for 1min, transferred into 4% sodium hypochlorite for 3min, and followed by rinsing in 70% ethanol for 30 seconds and finally washed thoroughly thrice in sterile distilled water to remove the traces of alcohol. These surface-sterilized bits were then dried on sterilized blotter paper.

Isolation of fungal endophytes from plant material

The surface-sterilized leaf bits were placed in Petri dishes containing sterilized Sabouraud dextrose agar (SDA) medium supplemented with streptomycin (100µg/ml) to suppress bacterial growth. Five segments were placed in one agar plate spaced at 2cm from each other and incubated at 25±1°C for 7 days in dark conditions and were regularly monitored to check the growth of endophytic colonies from the incubated segments. The hyphal tips of the developing fungal colonies were isolated and sub-cultured by transferring to fresh sterilized SDA slants and plates to maintain the pure cultures.

Identification of isolated fungal endophytes

Isolated endophytic fungi were morphologically identified based on their macroscopic examination (front and reverse side of fungal colonies, colony pigmentation) and microscopic characteristics (fruiting structures and spore morphology) under a bright-field microscope (Von Arx, 1981).

RESULTS AND DISCUSSION

Isolation of endophytic fungi

Total 40 edible leaf samples were collected from Kodagu District, Karnataka Table 1. The leaves were surface sterilized and were cultured on SDA plates. The cultures were incubated at 28^{o}C, and the mycelia of fungi emerged within a week assuming as non-endophytes were removed. The mycelia that emerged after two weeks were transferred to PDA plates and collected for endophytic culture stock. From 40 edible leaves, we obtained 85 pure endophytic fungal isolates which were considered as the starting material for, colony morphology, molecular characterization and further analysis.

Macroscopic and Microscopic Characters

The selected fungal endophytes were analysed by studying their various macro and micro morphological characteristics. Identification of fungal endophytes based on macro morphology involved observation of colony colour, colony texture, pigment production etc. while microscopic studies included shape and size of the spores, presence or absence of septa, number and type of septa etc. These morphological features were compared to the literature and the isolate was identified as species of *Alternaria*, *Fusarium*, *Tricoderma*, *Penicillium,* etc. Molecular based approaches have proved to be more useful in the taxonomical classification of fungi which are morphologically indistinguishable.

CHAPTER 4

Biological activity of endophytic fungi

INTRODUCTION

In most cases, secondary metabolites are created from intermediate compounds formed during primary metabolism. Sach was the first to recognize secondary metabolites in 1873, and they were referred to as natural products. Secondary metabolites are formed as a result of the interaction between endophytes and plants, which is governed by the endophytes' genes and the plant's environment. Endophytic fungi of all higher plants are becoming more and more popular as potential sources of physiologically active compounds (Selim *et al.,* 2012). Endophytic fungus produces a variety of natural products with distinct structures and bioactivities, which has huge potential for use in the medical, agricultural, and industrial fields (Zhang *et al.,* 2006).

Endophytes are chemical synthesisers inside plants, according to Owen and Hundley (2004). Endophytes that live on medicinal plants can provide bioactive secondary metabolites that can be used to treat a variety of ailments (Tejesvi *et al.,* 2007; Kumar *et al.,* 2014; Singh and Dubey, 2015). Many scholars have extensively examined a wide range of endophytic applications, including Jalgaonwala *et al.,* 2011, Kaul *et al.,* 2012, Bhardwaj *et al.,* 2014, Kaul *et al.,* 2014, Nicoletti and Fiorentino, 2015). Fermentation of endophytic fungi with bioactive chemical synthesis potential has various advantages, including repeatable and consistent productivity.

Antimicrobial agents, according to Guo *et al.,* (2000), are low molecular-weight organic natural compounds produced by microbes that are active against other microorganisms at low concentrations. According to Daljit Singh Arora and Navdeep Kaur 2018; Gopinath, *et al.,* 2017**;** Carbungco *et al.,* 2017; Igor *et al.,* 2016**;** Rajeswari *et al.,* 2014**;** Dhanalakshmi *et al.,* 2013 have reported the isolation of endophytic fungi having antimicrobial potential from *Moringa oleifera*. Where antimicrobial activity has the response surface methodology was effective which increased antimicrobial activity by 1.7-fold and good zone of inhibition was observed. Preeti Juyal *et al.,* 2017 and Suneel Kumar *et al.,* 2016 reported antimicrobial activity of dominant endophytes was checked against test bacterial cultures and showed potent antimicrobial activity against the test organisms and potent fungus was optimized for suitable media, pH, temperature, and salt concentration to obtain the maximum production of bioactive compound from *Fusarium oxysporum*. According to Rangari *et al.,* 2017; Avnish Kumar *et al.,* 2015 and Archana Nath *et al.,* 2014 reported biodiversity of endophytic bacteria and fungi in

the *Murraya koenigii* where the total antioxidant power and free radical scavenging activity of the fungal extracts were estimated using the total phenolic content and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay along with the flavonoids and alkaloids content and the antimicrobial activity of fungal extract were tested against three bacterial and one fungal strain, which showed maximum zone of inhibition.

MATERIALS AND METHODS

Production and extraction of secondary metabolites

The pure endophytic fungal colonies on SDA were cut into small pieces (6mm in diameter) and were then transferred into a 500mL Erlenmeyer flask containing 150mL of Sabouraud dextrose broth (SDB). All the flasks were incubated at 25±1°C for 21days with periodical shaking at 150 rpm. After incubation, the fermentation broth was filtered through a sterile muslin cloth, and to this filtered broth equal amount of ethyl acetate solvent was added in a separating funnel and agitated for 15min. The organic phase was collected, and the solvent was removed by evaporation under reduced pressure at 45^{o}C using a rotary vacuum evaporator. The concentrated crude extracts were then stored at 4^{o}C and were used for studying the biological activities (Raju and Victoria 2015).

Screening of bioactive properties in crude extracts of fungal endophytes

Dried fungal crude extracts were dissolved in DMSO to known concentrations and assessed for their bioactive potential.

Antibacterial activity of fungal crude extract

Four foodborne human bacterial pathogens were used for antimicrobial tests for all isolated fungal endophytes. Gram-positive (*Enterococcus faecalis)* and Gram-negative (*Escherichia coli, Salmonella typhi,* and *Shigella sonnei*) bacteria were procured from medical college, Yenepoya University, Mangalore, India. Freshly cultured bacterial colonies were used by sub-culturing in a nutrient broth medium for 24h. Each crude extract of endophytic strains was diluted by 1mg/mL using DMSO. The bacterial strains were swabbed on a sterile petri dish containing nutrient agar. Using sterile cork borer 6mm wells were made in which 100 µl of crude DMSO extract were loaded. Positive control was maintained by streptomycin 1mg/ml and DMSO was used as a negative control. The culture plates were incubated at 37ºC for 24h for bacterial growth. Antimicrobial activity was assayed by the measurement of any inhibition diameter zones.

Determination of minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

The minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) values were determined by the standard serial dilution assay (Julia Harumi *et al.*, 2019). All tests were performed in nutrient broth. Bacteria were cultured overnight at 37^0C. 100µl of liquid culture media and 10µl of bacterial suspension adjusted to 0.5 McFarland turbidity standard (~1.5×10^8 CFU/ml) and test sample dissolved in DMSO were distributed to each well in different concentration ranges from 0 to 100mg/mL. Plates were incubated at 37^oC for 24h. 15 µl of 0.01% resazurin was added to characterize cell viability. Then the plates were re-incubated for 4h, the absence of bacterial growth with the lowest concentration was taken as the MIC. Using the results of MIC, the concentrations showing a complete absence of visual growth of bacteria were identified, and 50µl of each culture broth were sub-cultured which showed no bacterial growth on agar plates after incubation for 24hat 37^0C in an incubator. The complete absence of growth on the agar surface at the lowest sample concentration was defined as MBC.

Antioxidant potential of endophytic fungal crude extract

DPPH free radicals' assay

The free radical scavenging activity for DPPH was measured according to Omar *et al.*, (2021) with minor modifications. 1ml of each extract was added to 3ml of methanolic solution of DPPH (0.3mM) and were shaken vigorously. This was followed by incubation for 30min in complete darkness at room temperature and then the optical density was measured at 517 nm with ascorbic acid as a positive control. The results are recorded as mean values.

FRAP (Ferric reducing/ antioxidant power) assay

Ferric reducing antioxidant power assay was measured according to the procedure described by Tuba and Gulcin, (2008) with some modification. Different concentrations of the samples (1ml) were added to 2.5ml of phosphate buffer (0.2M) and 2.5ml of 1% potassium ferricyanide. Each mixture was then incubated at 50°C for 20min and later 2.5ml of 10% trichloroacetic acid was added to each. The mixture was shaken vigorously, and the solution was mixed with 2.5ml of distilled water and 0.5ml 0.1% of $FeCl_3$. After 30min of incubation, absorbance was read at 700nm. Ascorbic acid was taken as a standard reference.

Phytochemical screening of crude extracts of fungal isolates

Phytochemical analytical tests were performed to detect the presence of chemical constituents such as phenols, flavonoids, alkaloids, terpenoids, saponins, tannins, glycosides, and steroids, according to the method described by Dipali *et al.,* (2013).

Total phenolic content (TPC)

The total phenolic content of the fungal extract was determined using Folin-Ciocalteu assay (Chang *et al.,* 2007). In this assay, the reaction mixture containing 1ml of the extract was added to 5ml of distilled water and later mixed with 500µl of the Folin-Ciocalteu phenol reagent. The mixture was agitated for 5min manually, and then 1ml of a saturated solution of sodium carbonate and 2 ml of distilled water were added while shaking to homogenize the dilution. After incubation in dark for two hours. The absorbance was measured at 765nm. The blank was prepared by substituting the same amount of diluted extract with methanol. Gallic acid was used as standard and expressed as µg/g gallic acid equivalent.

Total flavonoid content (TFC)

Total flavonoid content was determined by a colorimetric method Chang *et al.,* (2002). 1ml of the fungal extract was mixed with 5ml of distilled water and 0.3ml of sodium nitrate solution (5% w/v) was added to this mixture and then allowed to stand for 5min in dark. 0.6ml of aluminium chloride solution (10%) was added to the mixture and again kept in dark for 1minute. Consequently, 2ml of 1M NaOH was added to the mixture, and the total volume was made up to 10ml by distilled water and was mixed properly. After mixing the solution the tubes were observed for the development of yellow colour, the absorbance was measured at 510nm in a spectrophotometer.

Antidiabetic activity

Alpha-Amylase Inhibition Assay

The alpha-amylase inhibitory assay is the most used procedure to determine the antidiabetic activity; this was carried out using a modified procedure described by Singh and Kaur (2016). Fungal extract 150µl placed in a test tube and 100 µl of 0.2M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added and incubated for 10min. The reaction was initiated by adding 100µl starch solution at time intervals and then further incubated at room temperature for 10min. After incubation 200µl of dinitro salicylic acid (DNS) reagent was added. The tubes were then incubated in boiling water for 5min and cooled to room temperature. The reaction mixture was diluted with 2ml distilled water and the

absorbance was measured at 540nm using a spectrophotometer. A control was prepared using the same procedure replacing the extract with distilled water.

Glucose Diffusion Assay:

This assay was performed as described by Picot *et al.,* 2014 with minor modifications. 1 ml of 0.15 M NaCl containing 0.22mM D-glucose was loaded into a dialysis tube containing different concentrations (50- 200 µg/ml) of fungal extract and the dialysis tube was tied at both ends using clips and it was immersed in a beaker containing 45 ml of 0.15 M NaCl and 10ml distilled water. The control contained 1ml of 0.15M sodium chloride containing 22mM glucose and 1 ml of distilled water. The beakers were then placed on an orbital shaker and kept at room temperature. The diffusion of glucose into the external solution was monitored by every half an hour.

RESULTS AND DISCUSSION

Antibacterial activity

The ethyl acetate extract of all isolated endophytic fungi was studied on antibacterial activities with foodborne human pathogenic gram-negative and gram-positive bacteria (Abdou *et al.,* 1972) by well diffusion method. There are many reports on antimicrobial compounds produced by endophytic fungi in a culture that acts against plant and human pathogenic microorganisms (Santo and Silva 2015). The crude extract of endophytic fungi extracted using ethyl acetate displayed considerable antibacterial inhibition activity. The secondary metabolites of *Aspergillus terreus, Stemphylium vesicarium, Fusarium arcuatisporum, Neopestalotiopsis clavispora* and *Colletotrichum truncatum* have shown the inhibition against all the tested pathogens. The maximum zone of inhibition was recorded by *Fusarium arcuatisporum* against all tested bacterial pathogens *Escherichia coli*, *Salmonella typhi, Shigella sonnei* and *Enterococcus faecalis.* The results clearly demonstrate the broad-spectrum nature of the metabolite.

The isolate that showed high inhibition activity were further determined for their MIC and MBC by microdilution method. The major five isolates has more MIC and MBC value against *Escherichia coli, Salmonella typhi*, *Shigella sonnei* and *Enterococcus faecalis* Representing that endophytic fungus have therapeutic potential as they produce antimicrobial compounds.

DPPH (2,2-Diphenyl-1-picrylhydrazyl)

The scavenging potential of the fungal crude extracts were evaluated ascorbic acid was used as standard to assess the potential DPPH radical scavenging activity of endophytic fungal

extracts. Ethyl acetate extracts of all 85 fungal isolates exhibited scavenging activity among them five major isolates showed high scavenging activity.

Ferric Reducing Antioxidant Power (FRAP)

Reducing ability of fungal extracts was compared with ascorbic acid and was found to be inferior to ascorbic acid. The results obtained in the FRAP assay indicated good activity in all the isolates and the value is directly proportional to its antioxidant capacity which displayed a wide variation of reducing power assay.

Phytochemical screening of crude extracts of fungal isolates

Ethyl acetate extract of all 85 fungal isolates were evaluated for their phytochemical constituents using standard procedures. Different endophytes have shown the presence of different phytochemicals such as saponins, flavonoids, phenols, alkaloids, tannins, etc. In the present study, the phytochemical analysis of 85 fungal extract revealed the presence of different phytoconstituents.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The total phenolic content of the fungal extract was determined using the Folin-Ciocalteu reagent and was expressed in terms of gallic acid equivalent. All 85 fungal extracts exhibited enhanced levels of phenolic content.

Total flavonoid content of the fungal extract was expressed in terms of quercetin equivalent. All 85 fungal extracts exhibited enhanced levels of flavonoid content.

Antidiabetic activity

Alpha-Amylase Inhibition Assay

To check the antidiabetic activity alpha-Amylase inhibitory assay were performed for all the 85 fungal extracts each endophytic fungal isolates inhibited the enzyme at different rates. Among the 85 endophytic fungal extract five major isolates were considered due to high percent of inhibitory activity of the enzyme

Glucose Diffusion assay

Glucose diffusion assay was conducted to investigate the level of inhibition of glucose movement by the plant extract at various intervals of time which was assayed and compared with the control in the absence of plant extract. 85 fungal isolates with respect to its glucose retardation activity across the dialysis tube exhibited a significant inhibition effect compared to the control. However, the inhibition effect exhibited by the extract was significantly decreased the glucose movement across the membrane when compared to the control.

CHAPTER 3

Molecular sequencing

Introduction

Identification of the endophytic fungi is usually done based on conventional classification. The physical traits of endophytic isolates are useful for identifying and classifying fungi, whether they reproduce sexually or asexually. Traditionally fungi are also identified depending on cultural characteristics such as colony morphology in addition to sexual structures and spores. The isolation of endophytic fungi frequently results in the production of non-sporulating isolates (Guo *et al.,* 2000, 2008; Photita *et al.,* 2001; Cannon and Simmons, 2002; Kumaresan and Suryanarayanan, 2002; Wang and Guo, 2007; Sun *et al.,* 2008). These isolates present a unique challenge because they are unable to be assigned to a specific taxonomic (Lacap *et al.,* 2003). Endophytes can produce secondary metabolites that have biological functions, and they can contribute to the classification of the endophytes (Azevedo *et al.,* 2000; Schulz *et al.,* 2002). By recognizing the potential of endophytic fungus to create a wide range of bioactive compounds and the availability of unknown microbial diversity, studies were planned to extract and screen endophytic fungi from edible leafy greens in search of new bioactive metabolites. To verify the conventional morphological identification of the chosen species, the ribosomal RNA of some of the selected endophytic fungus isolated from the edible leafy greens has been sequenced.

Materials and Methods

Molecular identification of potential fungal endophytes

Fungal endophytes were selected for genomic DNA extraction due to its promising biological activities which were notably higher than the rest of endophytic fungal crude extracts. The genomic DNA was extracted using Expure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd., as per manufacture instructions. The fungi were identified using universal primers pairs targeting the internal transcribed spacers (ITS) regions of the ribosomic DNA, including ITS1F (5’ AGAGTTTGATCTGGCTCAG 3’) and ITS4R (5’TACGGTACCTTGTTACGACTT 3’). By PCR using the universal primers ITS5 and ITS4, the fungal fragments were amplified. The PCR conditions were: Initial denaturation 95^{o}C for 2min, denaturation 95^{o}C for 30se, annealing 55^{o}C for 30sec, extension for 72^{o}C for 1min, Final extension for 72^{o}C for 10min and finally Hold at 4^{o}C. The reaction products were separated by 1% agarose gel, and the amplicons were purified by a gel band purification kit. The purified

PCR product was then sent to sequences for BLAST analysis to estimate the phylogenetic relationships by using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The sequence was compared with those sequences already available in National Centre for Biotechnology Information (NCBI) GenBank database using BLAST search and CLUSTAL X software was used to generate phylogenetic tree alignment (Tamura *et al.*, 2007).

Chapter- 5

Characterization of bio-active compounds of endophytic fungi in edible greens.

INTRODUCTION

Chemical defences of plants are frequently associated with a group of closely related chemicals that provide protection against a variety of species. Endophytic microbes, which live in plant tissues without causing immediate harm or displaying overt disease symptoms, are potential sources of novel natural compounds with significant promise for therapeutic discovery (Strobel, 2006; Zhao *et al.*, 2010; Qadri *et al.*, 2013). Natural-source drugs play a critical role in the prevention and treatment of human diseases. According to Cragg and Newmann (2005), approximately 60% of novel medications created in the last two decades of the twentieth century were derived from natural ingredients and were successful in treating infectious disorders and cancer.

Natural products continue to be a reliable source of therapeutic leads, with microorganisms responsible for more than 40% of novel chemical entities (NCEs) identified between 1981 and 2005. (Khosla, 1997; Clardy and Walsh, 2004; Sieber and Marahiel, 2005). Furthermore, natural products or natural product derivatives account for more than 60% of anticancer and 70% of antibacterial medications currently in clinical trials (McAlpine *et al.*, 2005). In addition to their antibacterial and antifungal capabilities, endophytes bioactive chemicals can inhibit viruses and have antioxidant, anticancer, antidiabetic, anti-inflammatory, and immunosuppressive properties (Joseph and Priya, 2011). The structural diversity of endophytic fungi metabolites is vast, and these natural products have been divided into several categories, including alkaloids (e.g., amines and amides, indole derivatives, pyrrolizidines, and quinazolines), steroids, terpenoids (sesquiterpenes and diterpenes), isocoumarins, quinones, flavonoids, phenylpropanoids, lignans (Tan and Zou, 2001 and Yu *et al.*, 2010).

The structure of the molecules can be obtained from certain specific absorptions in the infrared region (IR) which indicates the presence of specific chemical characteristics (Pavia *et al.,* 2009). For structural elucidation, 13C and 1H NMR investigations are exploited to elucidate the chemical structure of a pure metabolite. Alternative medicines have grown in popularity in recent decades as a result of the important role natural source pharmaceuticals play in the prevention and treatment of human diseases. The importance of endophytes in the synthesis of novel structures for use in medicine has gotten a lot of interest since the discovery of taxol from the endophytic fungus Taxomyces andreanae. Plants with unusual environmental situations, particularly those with various biodiversity may have the ability to produce novel chemicals.

MATERIALS AND METHODS

GCMS analysis of crude extracts of endophytic fungal isolates

The identification of chemical compounds present in the extract was performed by comparing the mass spectra with data from NIST (2008) library. All identified components were summarized in terms of relative peak area percentage.

Cytotoxicity assay (Allium test)

Small bulbs (1.5–2.0 cm in diameter) of the common onion, A. cepa, (2n = 16) were purchased at a local supermarket. For each extract sample, a series of onion bulbs were placed in tap water (pH 7.3) for 48 h and then onion roots were treated with the leaf's extracts of *Alternanthera sessilis*. The test tubes were kept in an incubator at 22 ± 1◦C visible morphological modifications, such as changes in root consistency and colour as well as the presence of swelling (c-tumors), hooks or twists in the roots were observed.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR was used for the detection of the different functional groups present in the methanol crude extract. The FTIR was handled by the diffuse reflectance technique in which the dried fraction sample was assorted with potassium bromide to form a very well powder and then compressed into a thin pellet. The pellet was used for the analysis of different functional groups. The samples were irradiated by a broad spectrum of infrared light and the stage of absorbance at a meticulous incidence was plotted after. The absorbance was measured between 400-600 nm for the identification and quantification of organic groups.

NMR (Nuclear Magnetic Resonance)

The number of protons and carbon atoms presented in the purified compound was recorded through NMR spectral study (H^1 and C^{13}) recorded on *JEOL ECZ 400 high resolution, multinuclear ft-nmr spectrometer at* 400MHZ, respectively. The chemical shifts were measured in δ scale in ppm and are referenced to the residual proton in the NMR solvent.

SUMMARY AND CONCLUSION

India has a vast heritage of diverse ethnic groups and rich biodiversity with a great emporium and a treasure house of ethnobotanical wealth. In India, a large section of the rural populations living far away from the urban area still relies on traditional herbal medicine for their primary health care needs. This is because medicinal plants are easily available natural products and are cost-effective. India's leafy greens have their incredible diversity with dense nutrients, intake of leafy greens helps to fight against many health-related problems as it is blessed with an array of phytochemicals, antioxidants, vitamins, and minerals like beta-carotene, lutein, and zeaxanthin which protect our cells from damage and also helps in alleviating the age-related problems. Previously these leafy vegetables were restricted among the rural community of certain geographical areas but now a day a widespread network can be seen in markets of urban areas selling with the higher price due to preference of more of green vegetables in their diet, increasing health consciousness and changing consumption pattern. Hence in the age of speeding up medical cost and their side effects, people are eagerly turning to natural medicines i.e., herbs.

The present study provides an exploration of diversity in edible greens, utilized by local inhabitants in the surveyed area. Where the plants belonging to different families were identified by local names used by local people and by referring standard texts for its botanical names further these leaves were isolated for endophytic fungi, where **589** fungal isolates emerged from the leaf segments placed on the SDA plate. Based on morphological and microscopic features **85** isolates were extracted using ethyl acetate solvent extraction method, the obtained crude extract were subjected to antioxidant, antibacterial, and antidiabetic assay. The five isolates (E1, G3, H3, N4 and U4) displayed potential antibaticaterial, antioxidant as well as antidiabitc activity. Those five isolates were subjected to 18S rDNA partial sequencing for species identification and the DNA sequencings were submitted to NCBI Genbank and accession numbers were gotten for the fungi species (*Aspergillus terreus*, *Stemphylium vesicarium*, *Fusarium arcuatisporum*, *Neopestalotiopsis clavispora* and *Colletotrichum*

truncatum). The GC-MS analysis were carried out to identify the active compounds in the five isolates.

The H3(*Fusarium arcuatisporum*) isolate exhibited high activity in all biological assay and considered for further purification purpose by using preparative HPLC assay, 15 partically purifed fractions were collected. The biological screening was done for all the 15 partically purifed fractions where, the fractions (1 & 6) had the highest activity compare to the rest fractions. The fractions (1&6) were subjected to GC-MS, FTIR, and NMR analysis to identify the bioactive compounds.

This study demonstrate that endophytic fungi isolated from the studied edible leafy greens were rich sources for bioactive compounds and can be used in the pharmaceutical applications. Hence, there is a need for further investigation *in vivo* experiments to know more about their specific biological properties to approach novel natural products with possible applications in medicine, agriculture and in the pharmaceutical industry.

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Research paper publications

- **H. K. Tejaswini** & M. Jayashankar, "Exploration of Edible Leafy Greens Habitually Consumed in Kodagu District, Karnataka, India", International Journal of Current Research and Modern Education, Volume 6, Issue 1, Page Number 7-12, 2021.

- Jayashankar M, **Tejaswini H.K** and Mohammed Ali Saeed. Biological activity of *Stemphylium Vesicarium*: An endophytic fungus isolated from *Portulaca Oleracea*. World J Pharm Sci. 9(6): 175-183, 2021.

- **Tejaswini, H.K**, Jayashankar, M. and Mohammed Ali Saeed. Screening of Biological Effectiveness of Endophytic Fungi Associated with *Plectranthus amboinicus*: A focus on *Aspergillus terreus*. Int. J. Pharm. Sci. Rev. Res., 69(1): 24-31, 2021.

Conference and Workshop

- **Tejaswini H.K** and M. Jayashankar. Isolation and identification of endophytic fungi from edible greens and its antimicrobial activity. **Poster presented** in National conference on phytochemicals and microbial bioactive compounds- role agriculture and human welfare. Department of Microbiology and Biotechnology, Bangalore University, Bangalore. 3rd and 4th October 2019.

- **Tejaswini H.K** and M. Jayashankar. GC-MS analysis of bioactive metabolites from an endophytic fungus *colletotrichum truncatum* isolated from *Raphanus sativus* (radish) an edible leafy green. **Oral presentation** at 42nd annual conference of Indian association of biomedical scientists "IABMSCON-2021". Organised by CRL-KSHEMA and NUCSER, Nitte (Deemed to be university), Deralakatte, Mangaluru, India. 25th-27th November 2021.

- **Tejaswini H.K** and M. Jayashankar. Antibacterial, Antioxidant and Antidiabetic Assays of Endophytic Fungi Isolated from *Commelina benghalensis*. **Oral presentation** at International Conference on Advanced Biology. Organised by Inter University Centre for Evolutionary and Integrative Biology, University of Kerala, Kariavattom, Thiruvanathapuram. 23rd to 25th February 2022.

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Signature of the candidate Signature of the Guide